PRACTICAL MEDIA AND CONTROL MEASURES FOR PRODUCING HIGHLY TOXIC CULTURES OF CLOSTRIDIUM BOTULINUM, TYPE A[1]

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The wide assortment of preserved foods from which botulism has been contracted plainly indicates that *Clostridium botulinum* can utilize a variety of naturally occurring nutrients for production of toxin. Yet in the laboratory, meat infusion, peptone media are customarily employed for this purpose. When detailed studies on the purification and characterization of the toxin were contemplated, the expense of infusion media and the difficulty of obtaining meat during wartime presented serious difficulties. A search was, therefore, undertaken for media that could be prepared in quantity at minimum cost and yet provide the necessary nutrients for the production of highly toxic cultures of *Clostridium botulinum*, type A. A study was also made of certain biochemical changes that accompany toxin production, in order to provide, if possible, relatively simple and rapid methods for determining when to harvest cultures containing maximum levels of toxin. The results of this investigation, as reported herein, have provided means for obtaining sufficient toxin to permit crystallization and biochemical study of the pure toxin by Lamanna *et al.* (1946). A similar investigation of media for the preparation of toxoid was conducted concurrently by Nigg *et al.* (1946), who adopted somewhat different formulae to avoid the introduction of allergenic substances that might interfere with clinical use.

To a considerable degree the development of practical media was advanced by the unpublished contributions of a number of independent investigators whose names are listed in the acknowledgment. In addition to the unpublished data cognizance was taken of certain pertinent observations recorded in the literature, such as the work of Burke (1919), Elberg and Meyer (1939), Gladstone and Fildes (1940), Dack and Wood (1928*a*; 1928*b*), Dack, Wood, and Dehler (1928), Wagner, Meyer, and Dozier (1925), Dozier, Wagner, and Meyer (1924), Stark, Sherman, and Stark (1928), and Lamanna and Lewis (1946). The reviews on bacterial nutrition by Knight (1938) and Peterson and Peterson (1945) were also helpful.

METHODS

The "Hall" strain of *C. botulinum*, type A, obtained through the courtesy of Dr. J. H. Mueller, Harvard University, was selected for this investigation because unpublished work by McCoy and Sarles (1943) indicated that it produced more toxin per unit of culture than any other strain tested by them.

Inocula for experimental work were prepared by first transferring approxi-

[1] Studies conducted at Camp Detrick, Frederick, Md., from June to December, 1943.

mately 0.1 ml of the turbid supernatant fluid from a well-stirred meat mash stock culture to a tube or bottle containing a medium similar to those being tested. After incubation at 34 C for 18 to 30 hours appropriate aliquots were transferred aseptically to experimental media. Initially inocula were sometimes taken directly from meat mash cultures to test media; however, this introduced the possibility of carrying over unknown ingredients which would influence the results. Final data were obtained with inocula from cultures which had undergone several serial transfers in appropriate liquid media at 24- to 48-hour intervals.

The corn steep liquor employed in many experiments was obtained in wooden barrels from the Corn Products Refining Company, Argo, Illinois. As received, it contained approximately 50 to 60 per cent solids. When media containing this material were sterilized in the autoclave, massive precipitates were encountered. To avoid this difficulty the corn steep liquor was diluted sufficiently with water to make a thin, free-flowing slurry, which was then adjusted to pH 8.4 to 9.0 with concentrated sodium hydroxide. The mixture was heated to boiling and clarified by centrifugation or filtration. Approximately 40 per cent of the solids were removed. The filtrate could then be incorporated in culture media with the formation of relatively little precipitate upon sterilization. The quantities of corn steep liquor used in this investigation have been recorded in terms of the approximate amounts of total solids.

When casein or powdered milk were to be used, 10 per cent suspensions were first prepared by adding each 10-g lot of powder to 90-ml portions of tap water, adjusting to pH 7.6 to 8.0 with N/1 NaOH, and stirring in a Waring "blendor" until finely dispersed, stable suspensions were obtained. When necessary, the total volumes were brought to 100 ml by further additions of water. Appropriate aliquots of the 10 per cent suspensions were then mixed with other ingredients to give the desired final concentration of casein or milk.

Test media were usually prepared in 100- to 150-ml amounts in 6- or 8-oz screw-capped prescription bottles. The hydrogen ion concentration was adjusted with sodium hydroxide or hydrochloric acid so that the media were at pH 6.8 to 7.4 after sterilization in the autoclave at 120 C for 15 to 20 minutes. The bottles of media were inoculated soon after preparation with relatively heavy inocula (usually 2 to 5 per cent by volume) and were incubated at 34 C. Under these conditions no special procedures were required to insure adequate anaerobiosis, provided agitation was minimized to avoid undue aeration of the medium.

Growth was estimated by turbidity, evolution of gas, proteolysis, and microscopic observation of films stained by Gram's method.

For the determination of toxicity, cultures were first diluted in a solution of the following composition:

Gelatin (Difco)	2 g
$NaH_2PO_4 \cdot H_2O$	7.25 g
Na_2HPO_4	3.70 g
Distilled water to make	1,000 ml
Approximate pH	6.5

White mice weighing 18 to 24 g were then injected intraperitoneally with 0.5-ml aliquots of each dilution. Because of the limited number of animals available, only two to four mice were injected with each dilution, and statistically significant LD_{50} values could not be obtained. For this reason the data have been recorded in terms of an approximate MLD based on the greatest dilution causing death of half or more of the mice within 4 days.

Determinations of pH were made with a standardized glass electrode apparatus. Chemical analyses were performed according to the procedures given by Hawk and Bergeim (1942). Tests for reducing substances and nonprotein nitrogen were made, respectively, by the Folin-Wu and Koch-McMeekin methods.

RESULTS

Selection of Ingredients for Production of Toxic Cultures

Preliminary comparison of casein, gluten, and peptone. The initial trials were directed toward a preliminary evaluation of different protein sources to replace the casein digest and meat peptone commonly employed in media for *C. botulinum*, type A. A basal medium containing yeast extract, cerelose (commercial glucose), thioglycolic acid, and tap water was used to compare casein, corn gluten, Difco peptone, and pepticase (casein digest). When inoculated lightly, the various culture media listed in table 1 showed appreciable differences in toxin production. The basal medium without proteinaceous material or with 1 per cent Difco peptone failed to support growth. Media containing either casein or corn gluten in concentrations of 2.5 per cent allowed production of only 1,000 MLD of toxin per ml, whereas the pepticase medium supported the production of 100,000 MLD per ml.

When mixtures were employed, 2 per cent casein plus 0.5 per cent pepticase permitted the development of 1,000,000 MLD per ml. As the pepticase was decreased below this figure and the casein increased proportionally, toxin production was correspondingly reduced. Mixtures of casein and peptone allowed production of even less toxin than the casein alone. Because of physical difficulties of handling corn gluten in liquid media, less extensive trials were made with this material than with the casein, but it appeared from a single trial, using a combination of 0.1 per cent pepticase and 2.5 per cent gluten, that this mixture was equivalent to a similar medium in which casein replaced the gluten, for both produced 10,000 MLD of toxin per ml.

Since some toxin had been produced from the casein alone, it seemed possible that by increasing the inoculum or adjusting environmental conditions this protein might completely substitute for pepticase. A second series of experiments, was, therefore, conducted to compare casein digest with casein. In this case both the commercial product, pepticase, and a laboratory pancreatic digest of casein were employed in the basal medium mentioned above. After 3 days' incubation at 34 C, all three media contained approximately 100,000 MLD of toxin per ml. It seemed probable from these data that casein was as useful as casein digest for toxin production, thus eliminating the laborious digestive processes.

To determine the least amount of casein that would be practicable, varying concentrations from 0.05 to 2 per cent were tested. The results given in table 2 indicate that 0.25 to 0.5 per cent casein was adequate in the yeast extract cerelose medium previously described. In this range, 100,000 to 1,000,000 MLD of toxin per ml could be obtained regularly, and no further increase in yield was observed when the casein content was increased to 1 or 2 per cent. Lesser amounts than 0.25 per cent failed to support adequate growth, thus making abundant toxin production impossible. For routine toxin production 0.3 per cent casein has been used successfully.

TABLE 1

Variations in toxin production with culture media containing different kinds and combinations of proteinaceous material

SUPPLEMENTS TO BASAL MEDIUM* (%)				MLD PER ML × 10^5
Peptone	Pepticase	Casein	Gluten	
0	0	0	0	0†
1	0	0	0	0†
1	0	1.5	0	<0.01‡
0.1	0	2.4	0	<0.01‡
0.01	0	2.5	0	<0.01‡
0	2.5	0	0	1.
0	1.0	1.5	0	1.
0	0.5	2.0	0	10.
0	0.1	2.4	0	0.1‡
0	0.01	2.5	0	0.01‡
0	0	2.5	0	0.01‡
0	0	0	2.5	0.01
0	0.1	0	2.5	0.1

* Basal medium: Difco yeast extract 0.5 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.5. Dispensed in test tubes (10 ml per tube) and sterilized in the autoclave at 120 C for 15 minutes.

Supplements: Bacto peptone was obtained from Difco; pepticase (a tryptic digest of casein) received from Sheffield Farms, Inc.; technical grade of acid-precipitated casein was procured from Baker Chemical Company; gluten was a granular product made commercially from corn (manufacturer unknown).

Inoculum: One loopful of 5-day meat mash culture per tube.

Incubation: Three days at 34 C.

† No growth.

‡ Slight growth.

Supplements to replace yeast extract. To produce the quantities of toxin needed for biochemical studies, it was necessary to grow the cultures in large containers, such as 5-gallon carboys, and to have an adequate inoculum constantly available for successive production lots. Serial transfers of the culture in the production medium were therefore desired in order to provide a relatively large volume of inoculum at frequent intervals for the large culture vessels. Successive transfers in the casein, yeast extract, glucose medium resulted, however,

in partial loss of toxicity after the second transfer and eventual failure of growth after 4 to 6 transfers. From these results it appeared that the medium was deficient in some constituent necessary for the continued development of *Clostridium botulinum*.

A search was then undertaken to find supplements which would either replace or fortify the yeast extract. For this purpose a basal medium composed of 2 per cent casein, 0.6 per cent cerelose, and 0.05 per cent thioglycolic acid in tap water was employed. Serial transfers were made at daily intervals, using 2 per cent by volume of the preceding culture. Table 3 records the results of the first and fourth transfers after each had been incubated for 3 days at 34 C. The first transfer showed growth and toxin production to the extent of 100,000 to 500,000 MLD per ml in all media. After the fourth transfer appreciable differences in growth and toxin production were evident, depending upon the supplement employed. The basal medium without supplements, as well as the casein digest medium used to prepare the initial inoculum, showed no growth. Supplementing the basal medium with barley malt extract or beef infusion permitted slight growth but little or no toxin production. Yeast extract, ground beef, and barley sprouts extract supported some growth through the fourth transfer but permitted production of toxin only to the extent of about 100,000 MLD per ml. Corn steep liquor, cerophyl, or a mixture of yeast extract and cerophyl allowed adequate growth and production of 500,000 to 1,000,000 MLD of toxin per ml. Among the more useful of these supplements corn steep liquor seemed to be the most economical and readily available. For this reason it was used in later experiments to replace the yeast extract.

TABLE 2

Concentration of casein required for toxin production

CASEIN	MLD PER ML × 10^6	
	A	B
(per cent)		
2	10	5
1	10	1
0.5	10	5
0.25	5	1
0.10	*	*
0.05	*	*

Basal medium: Difco yeast extract 0.5 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.4. Sterilized in the autoclave at 120 C for 15 minutes.
Inoculum: Two per cent of a 24-hour culture in basal medium plus 2.5 per cent pepticase.
Incubation: Three days at 34 C.
Casein: Technical grade procured from Baker Chemical Company.
A and B: Indicate replicate trials.
* Growth slight or absent; no toxin formation detected.

Elimination of thioglycolic acid. Up to this point, thioglycolic acid had been

incorporated into the medium as a reducing agent to aid the initiation of growth, but there was no proof that it contributed beneficially to the medium. Comparison of casein, corn steep liquor, glucose media with and without 0.05 per cent thioglycolic acid showed, in fact, that abundant growth and toxin production to the extent of 500,000 MLD per ml could be obtained either with or without this reducing substance when 2 per cent of an actively growing culture was used as the inoculum. Although this constituent was periodically included

TABLE 3

Influence of supplements on toxin production in casein, glucose, thioglycolate medium

SUPPLEMENTS	MLD PER ML × 10^5 AFTER SERIAL TRANSFERS INDICATED	
	1st	4th
(per cent)		
None	1	<1*
Yeast extract (Difco) 0.5	5	1
Corn steep liquor (clarified) 0.4 (solids)	5	5
Cerophyl† 0.5	1	5
Cerophyl 0.05	1	1*
Cerophyl 0.25 + yeast extract 0.25	5	10
Ground lean beef 0.5 (wet weight)	1	1*
Beef infusion 2.5 (based on wet weight of meat)	1	<1*
Barley malt extract 0.25 (based on weight of malt)	1	<1*
Barley sprouts extract 0.25 (based on weight of sprouts)	1	1
Casein digest‡ 2.5 (based on weight of casein)	5	<1*

Basal medium: Casein 2 per cent, glucose (cerelose) 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water. Adjusted to pH 7.4. Sterilized in the autoclave at 120 C for 15 minutes. Readjusted to pH 6.8 to 7.2 with N/1 NaOH when necessary.

Inoculum: Media initially seeded with 2 per cent of a 24-hour culture containing peptic-case 2.5 per cent, Difco yeast extract 0.5 per cent, glucose 0.6 per cent, thioglycolic acid 0.05 per cent, and tap water.

Incubation: Three days at 34 C.

* Growth slight or absent.

† Dry, powdered, green vegetation commercially prepared.

‡ Formula as for inoculum above.

in other test media, it was eventually abandoned because toxin production was not influenced by its absence.

Selection of proteinaceous material. Using the corn steep liquor, cerelose basal medium, various protein materials were again compared with particular regard to their toxin-producing qualities. Table 4 shows that casein digest, casein from three different commercial sources, corn gluten, and powdered milk were equally satisfactory in that all of them permitted development of approximately 500,000 MLD of toxin per ml. Corn gluten was again regarded as undesirable because

of its insolubility in liquid medium. Casein required careful manipulation to disperse it properly in the medium. Casein digest, though more easily handled, was available only in limited amounts and at high cost. Powdered milk had the advantage of ready availability, minimum processing requirements, and easy dispersion in the culture medium. For these reasons the medium composed of powdered milk, corn steep liquor, and glucose was selected for various additional tests to determine the minimum concentration of each ingredient required, the effect of supplements, and the possible influence of varying the amount of inoculum.

Although powdered milk was chosen for further experimental work there was no indication that the use of casein was fundamentally objectionable. Possibly elimination of the additional constituents of milk, especially lactose, would

TABLE 4

Equivalent toxin production in media containing milk, casein, or gluten

PROTEIN SOURCE	CONCENTRATION	MLD PER ML × 10^3	
		A	B
	(per cent)		
Casein digest (pepticase)	2.5	1	5
Casein (Baker)	2.0	5	5
Casein (Hercules)	2.0	5	5
Casein (Coleman & Bell)	2.0	5	5
Powdered skim milk	2.0	5	10
Corn gluten	2.0	5	5

Basal medium: Alkali-heat-treated corn steep liquor 0.4 per cent dry weight, glucose (cerelose) 0.6 per cent dry weight, thioglycolic acid 0.05 per cent by volume, and tap water. Adjusted to pH 7.5. Sterilized in autoclave at 120 C for 15 minutes. Adjusted after sterilization to pH 6.8 to 7.2 when necessary with N/1 NaOH.

Inoculum: Two per cent by volume of a 24-hour culture in basal medium plus 2 per cent casein.

Incubation: Three days at 34 C.

A and B: Indicate replicate trials.

tend to discourage rapid growth and acid production by contaminants that occasionally find their way into mass cultures. In any event, either casein or powdered milk was as satisfactory as digests of casein or meat for the production of highly toxic cultures of the "Hall" strain, which is actively proteolytic and is, therefore, capable of decomposing protein as the culture develops.

Minimum effective concentrations of selected constituents. Table 5 summarizes the data concerning the minimum concentration of each ingredient required for maximum toxin production. All media permitted growth, but toxin production was maximum only when proper concentrations of all three constituents were used. This suggests that the requirements for growth and toxin production may not be identical in the case of *C. botulinum*. The omission of any one of the three ingredients prevented the accumulation of toxin in abundance, and when suboptimal amounts of various constituents were employed, toxin production de-

creased accordingly. The following are the approximate minimum concentrations which allowed maximum toxin production: 2 per cent powdered skim milk, 0.2 per cent corn steep liquor (total solids), and 0.3 per cent glucose. Inasmuch as the levels did not seem to be critical in the range of excess quantities,

TABLE 5

Minimum effective concentrations of milk, glucose, and corn steep liquor for toxin production

CONSTITUENTS OF MEDIUM (PER CENT DRY WEIGHT)			MLD PER ML × 10^6
Milk*	Glucose†	Corn Steep‡	
6.0	0.5	0.4	5
2.0	0.5	0.4	5
0.5	0.5	0.4	<1
0.2	0.5	0.4	<1
0.05	0.5	0.4	<1
2.0	0.6	0.4	10
2.0	0.5	0.4	10
2.0	0.4	0.4	10
2.0	0.3	0.4	5
2.0	0.2	0.4	5
2.0	0.1	0.4	1
2.0	0	0.4	<1
2.0	0.3	0.8	5
2.0	0.3	0.4	10
2.0	0.3	0.2	10
2.0	0.3	0.1	5
2.0	0.3	0	<1
2.0	0	0	<1

Inoculum: Five per cent of a 24-hour culture prepared in the first medium listed in the table.

Incubation: Three days at 34 C.

* Powdered skim milk.

† Commercial quality sold under trade name, Cerelose. Sterilized separately and added aseptically to other constituents for this test.

‡ Heavy, fermented corn steep liquor was clarified and then used in amounts required to provide the total solids indicated.

the amounts of glucose and corn steep liquor were increased to 0.5 and 0.4 per cent, respectively, for the routine production of toxin.

Observations on added ingredients. It was well established, both from the literature and from experience gained during this investigation, that the toxin was relatively unstable when cultures were incubated for prolonged periods. One of the factors influencing the stability of the toxin is the change in hydrogen ion concentration resulting from the accumulation of metabolic products of the organisms. In the hope of finding materials which would tend to stabilize the toxin and allow greater accumulation in the culture medium, a variety of sup-

plements were added to the milk, glucose, corn steep liquor medium described above. After inoculation, cultures were incubated at 34 C, and aliquots were removed on the first, second, and ninth days of incubation. Determinations of pH and toxicity were made on each sample, and the results are shown in table 6.

TABLE 6

*Influence of supplements on production and retention of toxin by C. botulinum (type A) in a milk, glucose, corn steep liquor medium**

SUPPLEMENT†	DAYS	pH	MLD PER ML × 10^6
Lard stick	1	5.9	>5
	2	6.0	10
	9	6.3	5
Peanut meal	1	5.9	5
	2	6.0	10
	9	6.3	1
Black peat	1	6.0	>5
	2	6.2	25
	9	6.8	<1
Blackstrap molasses	1	5.8	5
	2	5.9	25
	9	6.2	5
Cerophyl	1	5.8	5
	2	5.9	25
	9	6.2	5
Distillers' solubles	1	5.8	>5
	2	5.8	10
	9	6.2	5
None	1	5.8	5
	2	5.9	10
	9	6.2	5

Inoculum: Three per cent of a 24-hour milk, corn steep, glucose culture.

Incubation: 34 C.

* Basal medium: Dried skim milk 2 per cent, alkali-heat-treated corn steep liquor 0.3 per cent (dry weight), technical glucose 0.3 per cent, and tap water. Adjusted to pH 7.6. Sterilized at 120 C for 15 minutes.

† Supplements suspended in water, sterilized at 120 C for 15 minutes, and added aseptically to basal medium to give a final concentration of 0.2 per cent.

During the first 24 hours a marked drop from pH 7.0 to pH 5.8 to 6.0 occurred, and, in some cases at least, relatively large amounts of toxin accumulated. During the following 24 hours the pH remained unchanged or reverted slightly toward the alkaline side, and toxin production appeared to have reached the maximum level in most cultures. The basal medium contained 1,000,000 MLD

of toxin per ml, and those cultures containing supplements had toxicities of the same order of magnitude, although the presence of cerophyl, blackstrap molasses, or black peat tended to give slightly higher values. By the ninth day of incubation the pH of most media had reverted to 6.2 to 6.3, although the ones containing black peat had risen to pH 6.8. The toxicity of all cultures had decreased until 500,000 MLD per ml or less remained. In the case of black peat, which showed the marked rise in pH, less than 100,000 MLD of toxin remained. These results indicate that the supplements tested were of no special value for enhancing the stability of toxin or markedly increasing the yield. In fact, it appeared that additional constituents might make the medium less

TABLE 7

*Influence of agitation and amount of inoculum on toxin production in milk, glucose, corn steep liquor medium**

AGITATION†	INOCULUM‡	FINAL pH§	MLD PER ML × 10^8 AT DAYS INDICATED		
			1	2	3
	per cent				
None	10	6.5	1	10	10
None	5	6.7	1	10	5
None	2	6.2	1	10	10
None	1	5.9	—	—	10
None	0.5	6.0	—	—	10
None	0.1	5.9	—	—	5
4–6 times	10	6.5	<1	<1	<1
4–6 times	1	6.7	—	—	<1
4–6 times	0.1	5.9	—	—	10

* Powdered milk 2 per cent, alkaline, heat-treated, filtered corn steep liquor 0.4 per cent, and glucose (cerelose) 0.5 per cent, mixed with tap water and adjusted to pH 7.4. Medium sterilized in the autoclave at 120 C for 15 minutes and then readjusted to pH 7.0 when necessary.

† Manual shaking, as commonly used for mixing cultures with dilution blanks, was applied during each working day but not at night.

‡ Sixth serial transfer in the foregoing medium was used after 24 hours of incubation.

§ Determined with glass electrode apparatus after 3 days of incubation at 34 C.

desirable for toxin production because they added inert material, and in one case at least, the final pH of the culture was so altered that the toxin disappeared more rapidly than in the basal medium.

Influence of Inoculum and Agitation on Toxin Production

The fact having been established that the milk, glucose, corn steep liquor medium provided nearly optimum conditions for toxin production, a brief study was then made of the influence of agitation and of varying amounts of inoculum upon the rapidity of formation and final yield of toxin. Different lots of the medium were inoculated with 10, 5, 2, 1, 0.5, and 0.1 per cent of a 24-hour culture representing the sixth serial transfer in the same medium. These were incubated

in a quiescent state at 34 C for 3 days. Duplicate cultures, which received 10, 1, and 0.1 per cent inocula, respectively, were agitated four to six times during each working day by shaking in a manner similar to that used for mixing cultures in dilution blanks. At night no shaking occurred. These were also incubated for 3 days at 34 C. Gross observations of the cultures indicated that those receiving 2 to 10 per cent inocula grew most rapidly, as judged by evolution of gas and proteolysis. These cultures were tested for toxicity at intervals of 1, 2, and 3 days after inoculation, but the remaining cultures were tested only on the third day. The results are shown in table 7.

Although the heavier inocula showed maximum toxin production in 2 days, they yielded no greater concentration of toxin at 3 days than the smaller inocula so long as the cultures were not disturbed during incubation. Even with 10 per cent inoculum, daily agitation prevented the accumulation of large amounts of toxin but did not inhibit growth. Only in the case of the agitated culture which received 0.1 per cent inoculum did sufficient toxin accumulate to be detected at the dilutions employed for animal injection. This culture was slow to initiate growth, and the period of rapid toxin accumulation apparently occurred during the night when the culture was not being disturbed. Thus agitation was not applied at the time when it would do most harm. From these results it would appear that even mild agitation, which permits entrance of air into the culture during the critical period of toxin accumulation, is harmful. On the other hand, the exact amount of inoculum is not critical in quiescent cultures, though some saving in time of incubation may be gained by using 2 per cent or more inoculum.

Biochemical Changes Associated with Toxin Production

Experience with the milk, glucose, corn steep liquor medium showed that subtle differences between batches of medium altered the rapidity of appearance, final yield, and stability of the toxin produced in the culture. Minor variations in the purity of the ingredients, method of preparation, quality of inoculum, and degree of anaerobiosis may have accounted for some of these unpredictable differences. Whatever the causes, a study of the biochemical changes accompanying toxin production in the culture seemed necessary in order to develop a method for predicting the time of maximum toxin accumulation. Direct measurement of toxicity in mice, requiring 2 or more days, was too slow for routine testing of cultures because marked deterioration of the toxin often occurred before the tests were completed.

In two series of experiments, the relationships of pH, nonprotein nitrogen, and reducing substances to toxicity were studied to learn how these factors varied in different media and whether biochemical tests could be used to follow toxin development.

Influence of corn steep liquor. The first series consisted of four lots of medium composed of 2 per cent powdered milk plus 0.3 per cent glucose, to three of which were added 0.8, 0.4, and 0.2 per cent corn steep liquor, respectively. The inoculum for each lot was 5 per cent by volume of a 24-hour culture grown in the

In the absence of corn steep liquor (figure 1A) no increase in toxin occurred during the entire 96 hours of incubation. The concentration of reducing substances showed only slight variation from the initial value. There was a tendency for the concentration to drop slowly and then to rise again to approximately the original level. The nonprotein nitrogen increased sharply from none to about 5 mg per 10 ml during the first 24 hours and then more slowly to give a final concentration of nearly 9 mg per 10 ml. The hydrogen ion concentration showed a rapid drop during the first 24 hours and thereafter leveled off at about pH 6.9, where it remained for the entire incubation period.

In the presence of corn steep liquor (figure 1B, C, and D) the behavior of the cultures was strikingly different. Between 12 and 24 hours a rapid rise in the toxin concentration occurred regardless of the amount of corn steep liquor in the various lots of medium. The maximum level was reached between 24 and 45 hours and thereafter declined gradually. The reducing substances declined rapidly from an initial level of 9 to 10 mg per ml to levels of approximately 5 to 6 mg per ml at about the interval when maximum toxin production occurred. The correlation of these two points was so marked that it would appear possible to determine the optimum time of harvesting toxic cultures by simply following at frequent intervals the concentration of reducing substances. After the low level of reducing substances was reached, a slight but consistent rise occurred during the later phase of incubation. The nonprotein nitrogen increased rapidly during the first 24 to 36 hours to different levels depending upon the amount of corn steep liquor present in the medium. Thereafter they rose more slowly. Hydrogen ion concentration increased sharply as demonstrated by the rapid drop from pH 7 to 5.6 to 5.8, which occurred during the first 24 hours. Thereafter the reaction gradually reverted toward the alkaline side, so that the final pH of all three cultures containing corn steep liquor was 6.7 to 6.9. The decline in toxin seemed to correspond roughly to this increase in alkalinity.

Influence of glucose. The second series of tests was conducted in a medium composed of 2 per cent powdered milk and 0.2 per cent corn steep liquor. To one portion of the medium no glucose was added, whereas the remaining three portions received 1.0, 0.6, and 0.3 per cent glucose, respectively. Each lot of medium received a 5 per cent inoculum as described for the preceding series. Aliquots were removed from all cultures at 6-hour intervals during the first 48 hours of incubation at 34 C, and at 12-hour intervals thereafter until 96 hours had passed. The same tests employed for the first series of trials, described above, were again performed on samples from the second series. The results are shown in figure 2.

In the absence of added glucose (figure 2A) a rapid but relatively slight rise in the toxin level occurred during the first 24 hours, which quickly dropped so that by 36 hours no more toxin remained than was initially added with the inoculum. Reducing substances first showed a slight and slow decrease, followed by a gradual rise to a level somewhat higher than the initial value of 4.1 mg per ml. Nonprotein nitrogen increased rapidly during the first 24 hours to about 9 mg per 10 ml and then leveled off at about 10 mg per 10 ml. The initial pH of the culture was 7.2 and the final pH was approximately the same. During the first

36 hours some drop in pH occurred to a minimum level of 6.7 which was followed by a period of reversion.

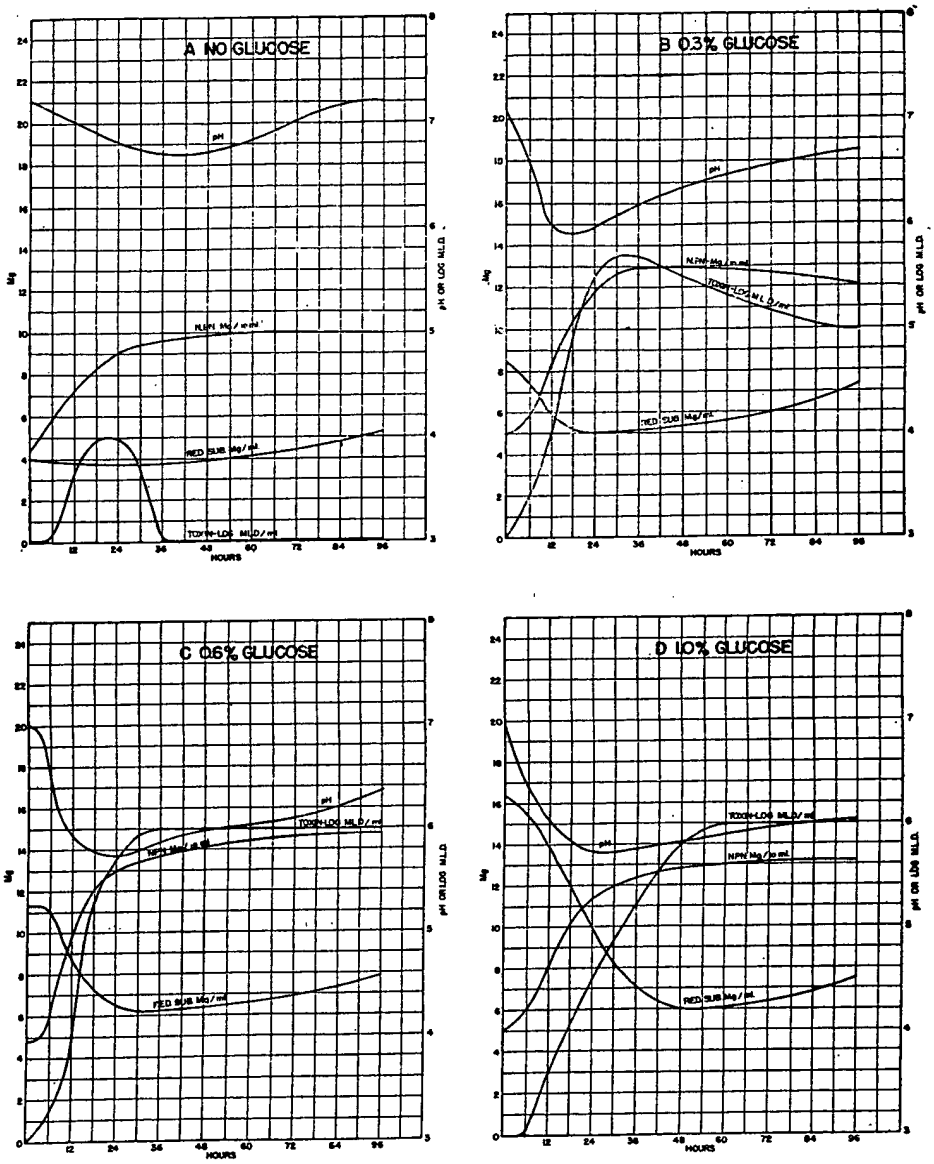
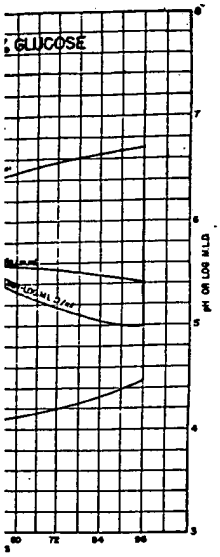
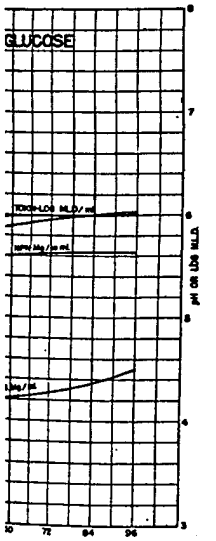


FIG. 2. CHANGES IN pH, NONPROTEIN NITROGEN (NPN), REDUCING SUBSTANCES (AS GLUCOSE), AND TOXICITY OF CLOSTRIDIUM BOTULINUM CULTURES IN MILK, CORN STEEP LIQUOR MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF GLUCOSE

As the concentration of glucose in the medium was increased (figure 2B, C, and D) the rapidity of the toxin accumulation became slower, but in all in-

which was followed





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stances reached a maximum level of 500,000 to 1,000,000 MLD per ml. Whereas the cultures containing 0.3 to 0.6 per cent glucose reached their maxima in approximately 30 hours, the culture containing 1 per cent glucose required nearly 60 hours. As in the preceding series of experiments, the reducing substances decreased rather sharply during the early phases of incubation from initial levels, which varied with the amount of added glucose, to low levels of 5.0 to 6.2 mg per ml at the time when maximum toxin production was reached. Here again the correlation between the minimum level of reducing substances and the maximum accumulation of toxin was so striking as to indicate the value of this chemical test for determining the optimum time for harvesting the toxin. The cause of the slow rise which characteristically occurred following the minimum level of reducing substances is unknown but presumably was related to the accumulation of protein degradation products, which possessed reducing characteristics. Nonprotein nitrogen increased rapidly from an initial level of 5 mg per 10 ml to approximately 13 mg per 10 ml during the first 24 to 36 hours of incubation and thereafter tended to level off. Although the most rapid increase in nonprotein nitrogen corresponded to the period of most marked toxin production, there was not the close correlation of critical values that has been noted in the case of reducing substances. In the presence of the various amounts of glucose a rapid drop in pH from approximately 7.0 to a minimum level of 5.7 to 5.9 occurred during the first 18 to 24 hours of incubation. In the presence of 0.3 per cent glucose a rather marked reversion to pH 6.7 occurred, but in the presence of 0.6 per cent glucose the final pH was approximately 6.4, and with 1 per cent glucose it rose only to about 6.0. Only in the first instance was there a marked decrease in toxin during the latter part of the incubation period, so that the stability of the toxin in the culture appeared to be closely related to the final pH.

Considering all the data presented in figures 1 and 2, it seemed that the rapidity of production, maximum level, and stability of the toxin were affected by the composition of the medium and the pH changes which occurred during incubation. The lack of corn steep liquor prevented full utilization of glucose, as indicated by the failure of reducing substances to decrease at the same rate or to the same extent as was observed in the presence of corn steep liquor. Likewise, the absence of glucose inhibited, to some extent, the utilization of proteinaceous materials, as indicated by the less extensive production of nonprotein nitrogen in this culture as compared to those with added glucose. In both cases toxin production was interfered with, yet growth occurred. When the medium contained all the ingredients in the proportions required for the production of large amounts of toxin, the characteristic changes in level of reducing substances were useful for determining when maximum concentration of toxin appeared in the culture. Although the nonprotein nitrogen was subject to characteristic alterations in level, it was not closely correlated with toxin production and was, therefore, of less value for following the progress of the culture.

It must be realized that the presence of contaminating organisms or the use of

media not suitable to toxin production so alter the culture that the changes in pH and reducing substance no longer bear the same relationship to toxin production. Nevertheless, in pure cultures of *Clostridium botulinum* a considerable measure of control can be exercised to ensure recovery of maximum toxin by the determination of pH and reducing substances throughout the growth cycle.

RECOMMENDED MEDIA

Many variations of the casein (or powdered milk), glucose, corn steep liquor medium have been used successfully for production of the toxin of *C. botulinum* type A. The following two formulae are suggested because they are easily prepared and effective for routine use:

Formula I (to make one liter)

Solution A. Suspend 20 g powdered milk in 180 ml of water. Add N/1 NaOH slowly as required to disperse the milk, until a stable suspension has been obtained by shaking or stirring. If necessary add water to make 200 ml.

Solution B. Add 6 g commercial glucose (cerelose) to a volume of clarified corn steep liquor equivalent to 4 g total solids and dilute to 800 ml with water.

Thoroughly mix solutions A and B, adjust to pH 7.4 to 7.6, dispense into culture bottles, and sterilize in the autoclave for 15 to 20 minutes at 120 C. After sterilization the reaction of the medium should be pH 6.8 to 7.2.

Formula II (to make one liter)

Solution A. Add 3 g casein (technical grade) to 50 ml water previously alkalized with 0.5 g NaOH per liter. Adjust to pH 10.5 to 11.5 with 10 N NaOH. Mix thoroughly with a mechanical stirrer for about 30 minutes. Readjust pH if necessary and stir again until a stable suspension is obtained.

Solution B. Add 5 g commercial glucose (cerelose) to a volume of corn steep liquor equivalent to 5 g total solids and dilute to 800 ml with water.

Pour solution A into solution B and rinse the container with 100 ml of water to remove remaining casein. Adjust the mixture to pH 7.2 to 7.4, bring the total volume to 1,000 ml with water, dispense into culture bottles, and sterilize in the auotclave for 15 to 20 minutes at 120 C. After sterilization the reaction of the medium should be pH 6.8 to 7.2.

For best results the media should be freshly prepared, inoculated with 2 to 5 per cent by volume of an actively growing culture of *C. botulinum*, type A, and incubated at 34 C. If aeration of the culture is avoided by minimizing agitation and using culture vessels with a low exposed surface area per unit volume, 500,000 to 1,000,000 MLD of toxin per ml can be obtained regularly in 48 hours or less.

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SUMMARY

Practical liquid media, composed of readily available and relatively inexpensive ingredients, have been developed for the production of highly toxic cultures of the "Hall" strain of *Clostridium botulinum* (type A).

The peptones usually employed in culture media can be replaced by 0.25 to 0.5 per cent casein (technical grade) or 2 per cent powdered skim milk.

Clarified corn steep liquor (0.2 to 0.4 per cent total solids) is a more adequate supplement than yeast extract for toxin production in casein or milk media, as demonstrated by relative viabilities and toxicities after 4 to 6 serial transfers in media containing these materials.

The presence of available carbohydrate in the form of 0.2 to 0.6 per cent commercial glucose (cerelose) markedly increases the total yield of toxin and enhances its stability in the culture medium by retarding reversion of pH toward the alkaline side.

The milk, corn steep liquor, glucose medium yields 500,000 to 1,000,000 MLD of toxin per ml of culture when inoculated with 2 per cent of an actively growing culture of *C. botulinum* (type A, Hall strain) and incubated in a quiescent state at 34 C for 24 to 48 hours. Small inocula or mild agitation tend to retard toxin production, and the latter may greatly inhibit its accumulation.

Characteristic changes in pH, concentration of reducing substances, and levels of nonprotein nitrogen occur during incubation of the culture. The relationships of pH and reducing substances to rapidity of accumulation and stability of toxin provide valuable control measures for securing the maximum yield of active toxin.

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